2019-07

Association of Ki-67, p53 and BCL-2 bio-markers expression with Clinic-Histopathology of breast cancer among women in Tanzania

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ASSOCIATION OF Ki-67, p53 and BCL-2 BIO-MARKERS EXPRESSION WITH CLINIC-HISTOPATHOLOGY OF BREAST CANCER AMONG WOMEN IN TANZANIA

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A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Master's in Life Sciences of the Nelson Mandela African Institution of Science and Technology

Arusha, Tanzania

July, 2019

ABSTRACT

Ki-67, p53, and BCL-2 are now emerging as markers for classifying breast cancer, guiding therapy and predicting treatment responses and prognosis. Restricted data currently exist on these molecular markers in Tanzania; hence, we assessed the expressions of Ki-67, p53, and BCL-2 and associated them with clinical histopathological features in breast cancer patients attending Muhimbili referral hospital in Tanzania. This retrospective cross-sectional hospital-based study was carried out between 2016 and 2017. For this research, only women were chosen with proven breast cancer, complete clinical history and accessible paraffin block samples. Tissue samples were immunohistochemically stained for Ki-67, p53, and BCL-2, with respect to their specific Monoclonal Mouse Anti-Human. The relationship between Ki-67, p53 and BCL-2 expressions and clinical histopathological features was determined using a multinomial linear regression model. Only 76 cases met the inclusion criteria for this study, with a mean age of 51.32 ±14.28 years. Of these, 86.4% were stage III and IV, whereas 83.5% cases had grade 2 and grade 3. Upon immunostaining, 85.5% and 57.9% were Ki-67 and BCL-2 positive, respectively. Log-linear analysis showed no statistically significant association among biomarkers expression and CH features. However, multinomial linear regression showed higher possibility for association between Ki-67+, p53- and BCL-2+ with age, grade, stage and tumor (T) stage. BCL-2 was positively correlated with Ki-67 expression contrary to p53, which was negatively correlated with BCL-2. Conclusively, there is evidence of correlation between the studied markers with CH features making these markers potential tools for evaluating treatment response in individualized therapeutic schemes.

AUTHOR'S DECLARATION

I, Hidaya Mansouri, declare that this dissertation is my own work. It is being submitted for the Master degree in Life Sciences, Nelson Mandela African Institution of Science and Technology. It has not been submitted for any degree or examination at any other University.

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Hidaya Mansouri

Name and signature of Candidate

24/07/2019

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Date

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CERTIFICATION

The undersigned certify that all supervisors have read and hereby recommend for acceptance by the Nelson Mandela African Institution of Science and Technology a dissertation entitled "Association of Ki-67, p53 and BCL-2 markers expression with clinic-histopathology of breast cancer among women in Tanzania" in fulfillment of the requirements for the degree of Master of life Science of the Nelson Mandela African Institution of Science and Technology.

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ACKNOWLEDGEMENTS

First and foremost, I thank Allah (SWT) for letting me alive to see this work achieved. Thank you for your guidance, strength, power of mind, protection, skills and for giving me healthy life.

I acknowledge my main supervisor, Dr. Elingarami S. Nkya for tirelessly guiding me through the academic process and professional advice in order to ensure that I have achieved the needful in a timely and friendly manner. I also thank the co-supervisor Dr. Emmanuel A. Mpolya, for coordinating the collaboration successively and for the scientific knowledge on statistical analysis and guidance whenever needed.

I acknowledge Dr. Leah F. Mnango and Mr. Erick Philip Magorosa, Senior Pathologist and Head of Histopathology Unit at the Central Pathology Laboratory (CPL), Muhimbili National Hospital (MNH) respectively, for having given me their guidance when I was mostly in need. I would also like to extend my gratitude to Dr. Henry Mwakyoma and all my colleagues and staff at the Department of Pathology of MNH, for their warmly welcome and collaboration, and for accepting my short stay.

I am grateful to the Tanzanian government, the Nelson Mandela African Institution of Science and Technology (NM-AIST), as well as its all staff members, for accepting me as citizen and student here. I am highly indebted to the World Bank Group and the Centre for Research, Agricultural Advancement, Teaching Excellence and Sustainability in Food and Nutritional Security (CREATES-FNS) project, for not only their unlimited financial support but also their friendly collaboration to undertake this programme and research despite the involved challenges

Special acknowledgement goes to my lovely parents and sister Mr./Ms./Miss Mansouri, as well as my acolyte for their unconditional support and accepting my long period of absence and for seeing me through the process. Thank you for your trust, it kept me going and alert.

Last but not least, I thank all my fellow colleagues from NM-AIST or elsewhere who have made the completion of this dissertation possible. May God bless you all.

DEDICATION

This work is wholeheartedly dedicated to my beloved parents Mr. Mansouri Ahamed and Mrs. Assiata Mohamed Soilihi, my sister Ms. Mansouri Kinaya as well as my partner Mr. Said Kamal who have been my source of inspiration and gave me strength when I thought of giving up, who continually provide me their moral, spiritual, emotional and financial support.

To my whole family for their undying support, their unwavering belief that I can achieve so much, who shared their words of advice and encouragement to finish this work.

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LIST OF ABBREVIATIONS AND SYMBOLS

NCDs	Non-Communicable Diseases
MIR	Mortality-to-Incidence Ratio
IHC	Immunohistochemistry
ORCI	Ocean Road Cancer Institute
MNH	Muhimbili National Hospital
BC	Breast Cancer
Ki-67	Nuclear antigen protein for cell proliferation
p53	Tumor suppressor antigen
BCL-2	B-cell lymphoma 2
LMICs	Low- and Middle-income countries
IDC	Infiltrating Ductal Carcinoma
H&E	Hematoxylin and Eosin staining
ILB	Infiltrating Lobular Carcinoma
CH	Clinical Histopathology
HR	Hormonal Receptors
ER	Estrogen Receptors
PR	Progesterone Receptors
HER-2/Her-2	Human Epidermal growth factor Receptor 2

CHAPTER ONE

INTRODUCTION

1.1 Background

As a heterogeneous disease with various features, previous studies have shown that breast cancer (BC) formation results from mutations and atypical changes in the genes responsible for modulating cells growth and upholding their health (Jagusch, 2010). Prompt detection of breast cancer plays clinical prominence, and can be used to make treatment decisions when adjuvant therapy is most probable to react with efficacy to clients. Despite the hostile price, efficient input, and scarcity of useful information for cancer management, biomarker test has shown a promising outcome in determining prognosis, and facilitating treatment planning for breast cancer management (Zaha, 2014).

Ki-67 is a nucleus protein originally identified in 1980s as a proliferative marker, merely identified in cellular division (G1, S, G2 and M-phase) (Li, Jiang, Chen & Zheng, 2015). Over the years, Ki-67 has been correlated with clinical and histopathological factors for prognosis of many cancers, including breast cancer. Furthermore, other biomarkers such as p53 and BCL-2 have also attracted attention from researchers. p53 is a tumor suppressor gene which normally limits cell development by monitoring quick cells division into new cells and rectifying DNA mismatched, but it is also a significant prognostic marker in early breast tumor screening. p53 expression has been demonstrated by diverse authors to be part of up to 50% of breast tumor through DNA impairment, oncogene activation, hypoxia, oxidase stress, viral infection (Shapochka, Zaletok & Gnidyuk, 2013). BCL-2 is responsible for promoting cell survival, inhibiting the action of pro-apoptotic antigen, and is expressively associated with hormone receptor status, because of its expression in ordinary breast glandular epithelium and estrogen-upregulated.

Molecular expression profiles for tumor markers such as Ki-67, p53 and BCL-2, are currently emerging as indicator for classifying breast cancers, guiding therapy, and forecasting treatment responses and prognosis (Sejal & Beiyun, 2011). However, biomarkers testing, especially for Ki67, p53 and BCL2 molecular markers are unfortunately not available or not routinely performed in many developing countries such as Tanzania, apart from hormonal receptor (HR); ER, PR and Her-2, which are routinely used to guide hormonal treatment choice.

To date, Tanzania has not yet carried out a population-based study that could give a true picture of the national cancer burden. Moreover, the associations (and their mechanisms) between prognostic factors for BC new biomarkers, such as Ki67, p53 and BCL-2 remain under examined as diagnostic test in the country. The current study aimed at examining Ki67, p53 and BCL-2 expressions as prognostic markers concomitantly associated with clinical and histopathological factors in Tanzanian patients with BC using immunohistochemistry technique in order determine to potential of these markers being use as tools for evaluating treatment response in individualized therapeutic schemes

1.2 Problem statement

BC has been and still remains an important chronic disease with major public health importance, causing preeminent global mortality and morbidity after cervical cancer. Over one million cases are identified with BC in which more 410 000 patients die annually in the world (Ghoncheh, Pournamdar & Salehiniya, 2016). Along these lines, Tanzania projected an amplified breast cancer cases of 2732 in 2012 to 4961 in 2030 (MoHCDGEC, 2017); whereas nearby 80% of these patients are likely to die. However, current status shows an unexpected rapid increasing amount of new cases contrary to the established projection (Fig. 1).

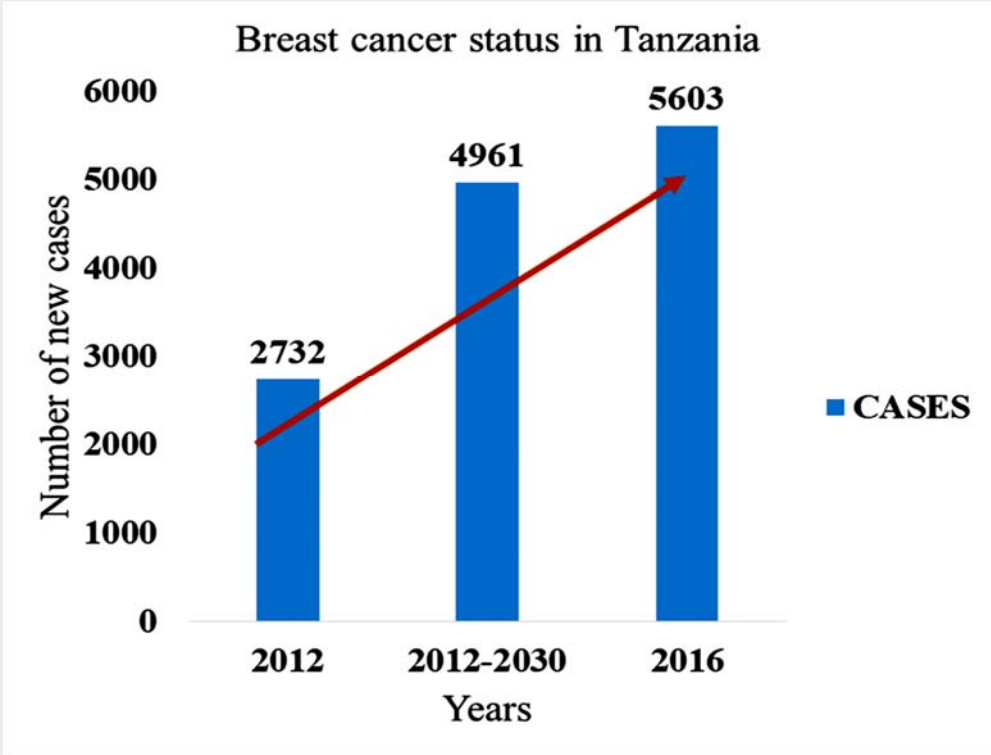

Figure 1: Estimated burden of breast cancer from 2012 to 2030 in Tanzania

(Source: Report from Ministry of Health, Community Development, Gender, Elderly and Children of Tanzania, March 2018)

Countless investigation linked the high incidence and mortality of disease to restricted diagnostic services, deficiency of effective treatment to those patients with BC and lack of information on additional bio-markers specially Ki-67, p53 and BCL-2 to guide treatment choice and possible prognosis. Moreover, prognosis of BC and treatment outcome has also been linked to high expression of prognostic markers such as Ki-67, p53, and BCL-2 (Angel, Carmen Del Rio, José, Michel & Álvaro, 2014; Mbonde, Amir, Akslen & Kitinya, 2001; Nalwoga, 2010). Unluckily, in many low-resource countries, including Tanzania, the assessment of bio-markers is either not available or not regularly used to guide therapeutic choice and possible prognosis (Silverstein, Sood & Costas-Chavarri, 2016). Understanding histological characteristics of breast cancer and their associated prognostic markers is therefore essential for determining prognosis and select suitable systemic therapy for patients. This will improve the treatment outcome and prove health of BC patients.

1.3 General objective

To assess BC biomarker expressions (Ki-67, p53 and BCL-2) and their associations with clinical histopathological features in designated breast cancer patients at Muhimbili National Hospital, Tanzania.

1.4 Specific objectives

(i) To define the clinic-histopathological characteristics and expression levels of Ki-67, p53 and BCL-2 using Immunohistochemistry in patients with breast cancer.

(ii) To associate Ki-67, p53 and BCL-2 expression levels with clinical histopathological features.

1.5 Research question

Does any link exist between Ki-67, p53, and BCL-2 bio-markers and the clinical histopathological features of breast cancer (stage, tumor size, type, grade and age) in Tanzanian patient breast cancer cohort?

1.6 Significance of the study

A better understanding of expression of Ki-67, p53, and BCL-2 associated to clinical histopathology feature of BC using sensitive and specific immunohistochemical (IHC) techniques will enhance BC classification, prognosis, as well as good therapeutic outcomes.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Breast cancer is one of the most prevalent cancer in African females and often diagnosed in advance phase, largely due to scarcity of timely detection and screening and being fatal among 56 per cent of Tanzanian women according to Cancer Care Foundation.

It is the third most frequent female cancer following cervix cancer. Further, it is the second most prevalent source of death amongst Tanzanian women (MoHCDGEC, 2018) (Fig. 2). While BC incidence rates are greater in developed countries than developing countries, lethality rates are excessively higher in developing countries (MoHCDGEC, 2017), owing to inadequate ability to implement prevention, early detection and therapeutic programs. The lifetime risk of BC among females in Tanzania is 1 in 20 and roughly half of all Tanzanian women with BC in Tanzania die of the disease (MoHCDGEC, 2017).

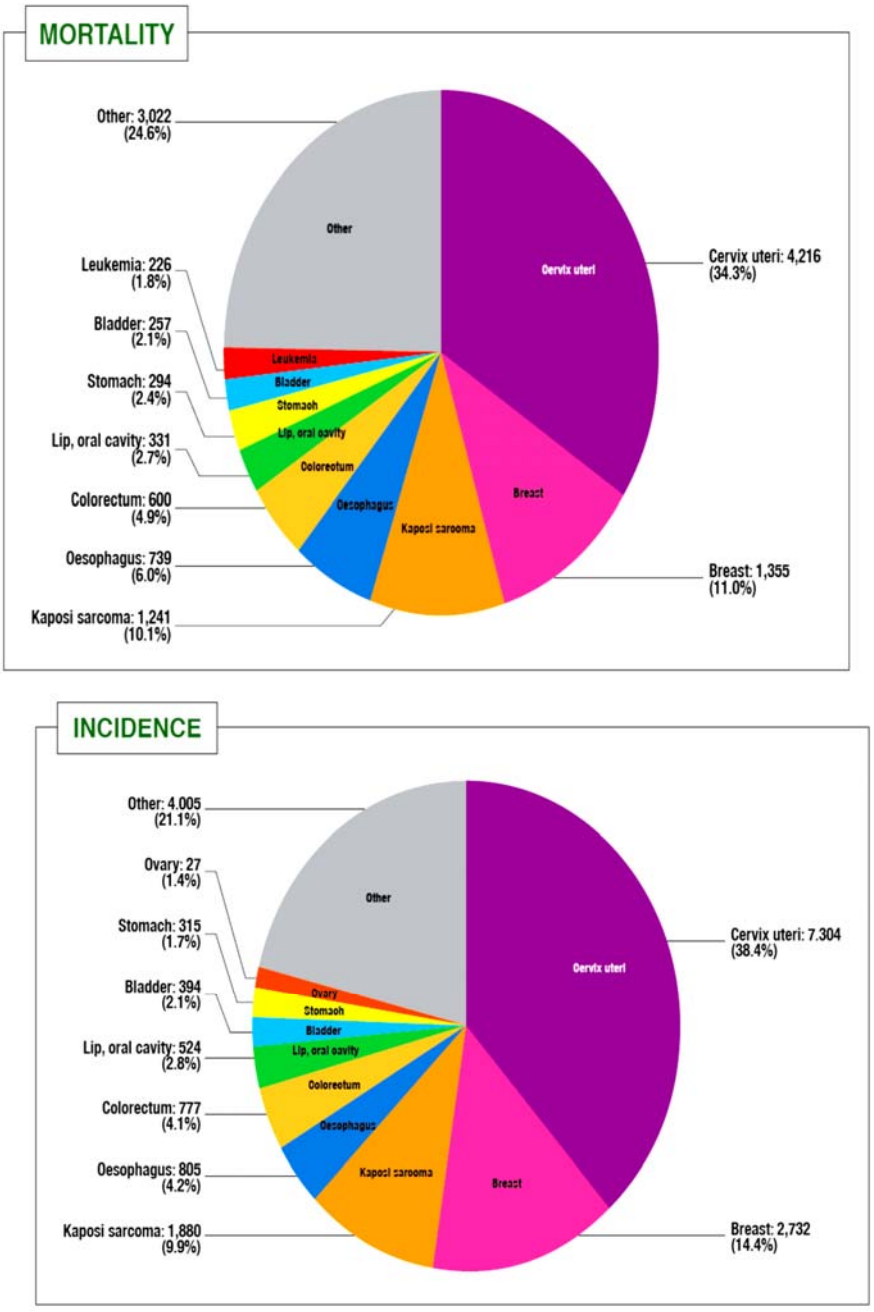

Figure 2: Distribution of cancer in Tanzania

Source: MoHCDGEC, United Republic of Tanzania (2018)

Histopathology, therapy reaction, metastatic models and result of breast cancer are heterogeneous (Eccles *et al.*, 2013; Kontzoglou *et al.*, 2013; Viale, 2012). Built on such elevated level of diversity, the disease cannot be considered as a separate clinical-pathological unit.

2.2 Association between tumor biomarkers and breast cancer

Tumor markers, also called bio-markers, are biomolecules found at upper/lower than normal levels in different samples such as blood, urine, or body tissue of some people with disease or cancer. Most of those bio-markers are often implicated in regulation of cell functions, like apoptosis, cell proliferation or cell surviving. One example of cancer markers is tumor suppressor genes, which may be down-regulated in cancer (Mantovani, Collavin & Del Sal, 2018).

Numerous studies have shown implication of Ki-67 as nuclear protein that serves as an indicator for cell proliferation, and has also been correlated with clinic histopathology and prognosis of many cancers, including BC (Shapochka, Zaletok & Gnidyuk, 2013; Strand *et al.*, 2013; Zaha, 2014). Other bio-markers like p53, BCL-2 have also attracted attention from researchers. p53 is a tumor protein (also called TP53) encoded by p53 tumor suppressor gene that normally limits cell growth by monitoring quickly dividing cells, restoring inconsistent DNA, and regulating apoptosis, but it is also a key prognostic marker in early detection of BC (Dumay *et al.*, 2013; Gasco, Shami & Crook, 2002). BCL-2 is an antiapoptotic cell protein encoded by the BCL-2 gene and has an imperative effect for promoting cell survival, inhibiting the action of pro-apoptotic antigen. BCL-2 is also associated with hormone receptor status, because of its manifestation in normal breast glandular epithelium and upregulated by estrogen. In addition, Ki-67, p53 and BCL-2 f are the most significant and helpful predictive variables presently accessible for endocrine treatment.

Therefore, it is necessary to evaluate a feasible connection between the manifestation of these biomarkers, clinical pathological condition and the risk of BC in Tanzanian women.

2.3 Data restriction on breast tumor markers test in Tanzania

One of BC's diagnosis constraints encountered in Africa is molecular characterization of tumor markers in patients. Several investigations have put forward that African BC is largely caused by overexpression of hormone receptors (Silverstein *et al.*, 2016). Pathologists and researchers working in low resource countries have been challenged as regards to the Standard Operating Procedures (SOPs) for IHC staining, due to many reasons, including outperformance of surgery, pre-treatment of tissue samples, poor quality specimens from large and necrotic tumors, doubtful quality of fixative materials, lengthy stay in fixative chemical (regularly for several weeks), poor laboratory techniques and quality

assurance/quality control practices, which often lead to inapplicability of advanced immunohistochemistry techniques (Kabel, 2017).

In Tanzania, a small number of studies are accessible on IHC performance in molecular classification of BC markers like Ki-67, p53 and BCL-2. In total, there are 169 district hospital and 30 regional referral hospitals in which four are public referral and zonal hospitals; Muhimbili National Hospital (MNH) for the coastal zone, Mbeya Referral Hospital (MRH) in the Southern highlands zone, Bugando Medical Centre (BMC) in the lake zone, and Kilimanjaro Christian Medical Centre (KCMC) in the Northern zone (MoHCDGEC, 2017). Unfortunately, not all healthcare activities related to BC care are conducted as planned compounded with the limitation of human resources and the supply of important health commodities. The four public referral and zonal hospital as well as Aga Khan Hospital (AGH) are the most prominent medical centres that offer cancer screening services. However, chemotherapy, and palliative therapy are only offered by the Ocean Road Cancer Institution (ORCI). Nevertheless, they lack diagnostic facilities such as IHC technique. Meanwhile, all cancer patients in the regional and peripheral zones are directed to MNH for HR and Her-2 examination or other IHC tests. This institution offers an original assessment of BC instances identified in Tanzania where they lack a population-based registries (Burson *et al.*, 2010; MoHCDGEC, 2017).

Burson *et al.* (2010) performed a two-year survey in Tanzania using medical record information from all breast cancer clients entering the Ocean Road Cancer Institute (ORCI) between July 2007 and June 2009.

Their research revealed that the frequency or molecular characteristics of breast cancer in Tanzania are little understood (Burson *et al.*, 2010). The MNH provides radio and chemotherapy for breast cancer patients in partnership with ORCI. However, this collaboration offers extremely specific treatment, but both clinics are overcrowded with lengthy waiting times for critical facilities as surgery and pathology assessment (MoHCDGEC, 2017), and therefore treatment outcomes are not really sensitive. Thus improving healthcare facilities for early diagnostic and cancer management is needed.

A 2017 study from the MoHCDGEC of Tanzania showed that the fraction of BC patients expressing hormone receptors in various medical centre could be substantial and significantly affected for therapeutic plans (endocrine therapy). Hence, the current queries are: how other prognostic bio-markers such as Ki-67, p53 and BCL-2 are expressed vis-à-vis age, stage and

grade in patients with BC in these specialized hospitals? Is there any correlation between expression of these other bio-markers and clinical histopathological features of breast cancer cases admitted at MNH?

2.4 Implication of Biomarkers test on personalized medicine

Bio-markers are usually split into prognostic, predictive and therapeutic response markers. Prognostic biomarkers enable the forecast of an external cancer's natural cycle, thus enabling the distinction between benign tumors and aggressive tumors.

The majority of the biological markers have been found through molecular profiling research relying on connection or interrelation between a molecular pattern with the diseases behavior and are used to evaluate the likelihood of a person being given a special therapy (personalized medicine).

Most bio-markers have been discovered by molecular profiling studies based on the association or correlation with molecular signature and disease behavior, and are used to evaluate the probability that a patient will benefit from a particular treatment (personalized medicine).

One of the initial molecular profiling studies reported by Golub et *al.* (1999) demonstrated that gene expression patterns could classify tumors, thereby providing new perspectives into pathological tumor, such as its stage, grade, clinical history, and therapeutic response (Golub *et al.*, 1999). Through scientific progress, immunohistochemistry was revealed to be a sensitive and specific technique used to identify biomarkers to gain knowledge on diagnosis and treatment outcome predictors (Duraiyan, Govindarajan, Kaliyappan & Palanisamy, 2012). Overall, these studies have highlighted the condition of Tanzanian (African) women with BC most of whom present the disease at progressive phase, as well as the influence of tumor markers on the expansion of BC in distinction to the rest of the developed country. Most health-related projects in Tanzania have focused on communicable disease such as tuberculosis, HIV, and female and child health ((WHO), 2016), which have reinforced the safety scheme and could also be leveraged to enhance breast cancer care.

In summary, improvements of BC management, including development of management guidelines, improvement of pathology amenities, and inclusion of immunohistochemistry for biomarkers assessment as a routine cancer management procedure, could have a progressive influence on the survival of persons with the disease, and may open further

prospects in the application of new technologies, such as nano-medicine, for personalized cancer management.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Study area

The present research was performed at the Muhimbili National Hospital's Central Pathology Laboratory in Dar es Salaam, Tanzania. MNH acts both as a Coastal Zone and as domestic referral and educational center for universities. It has a 1500-bed ability, supplying 1000 to 1200 weekly outpatients, and accepting 1000 to 1200 daily inpatients. It is the primary clinic with Diagnostic Laboratory Department, consisting of six facilities, as well as the Center Pathology Laboratory (CPL), which provides excellent engineering facilities (histology, hematology, clinical engineering and immunohistochemistry).

3.1.2 Patients and sample collection

This was a retrospective cross-sectional hospital based study in which patients were self-presented or referred from different hospitals to the department of pathology for general surgery. From 775 total patients, 76 out of 391 cases qualified for inclusion criteria for samples analyzed for Ki-67, p53 and BCL-2 biomarkers via immunohistochemistry (IHC). In addition, the selected patients were considered with primary tumor without neo-adjuvant treatment. Patient information was retrieved from data routinely reviewed and documented in cancer registry and electronic system. The exclusion criteria involved 384 patients who were either male patients with breast cancer, patients with a benign condition, patients with secondary breast cancer, missing block tissues and/or those lacking clinic-histopathological information (Fig. 3).

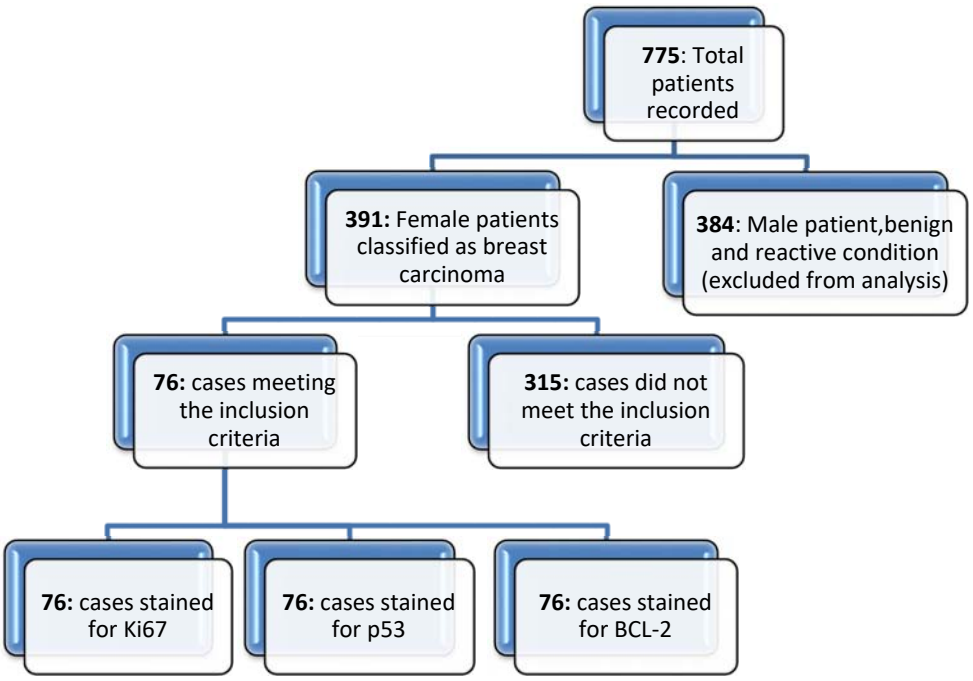

Figure 3: Distribution of patients and biomarkers categories across different pathological characteristics

3.1.3 IHC reagents

A microscope frosted slides, Dako kit with Monoclonal Mouse antibody of Ki-67 (Dako Monoclonal Mouse Anti-Human, MIB-1 ready to use), p53 (Dako Monoclonal Mouse Anti-Human, DO-7 ready to use) and BCL-2 (Dako Monoclonal Mouse Anti-Human,124), Target Retrieval Solution High pH (50x), Peroxidase-Blocking Reagent (ready-to-use), HRP (ready-to-use), DAB + Chromogen, Substrate Buffer as well as Wash Buffer (20x) were all provided by Labulax Supplies Limited (Nairobi, Kenya). Additional materials such as xylene and alcohol (100% - 95%) and other operative machine was providing at MNH.

3.2 Methods

3.2.1 Study design

This was a retrospective cross-sectional hospital based study which reviewed Hematoxylin and Eosin (H & E) slides, breast cancer registries, retrieved blocks from archives and conducted laboratory investigation on breast carcinoma tissue. IHC was applied to determine the expression of Ki-67, p53 and BCL-2.

3.2.2 Samples size estimation

The sample size was calculated with the formulation: $\boldsymbol{N = \frac{z^2 x\ p\ x(1-p)}{e^2}}$. Whereas N denotes the lowest sample size for tracking down a noteworthy results for an event and a fixed level of risk, z: Confidence level (the standard value of the 95% confidence level will be 1.96), p: 11% (MoHCDGEC, 2018) estimated proportion with the disease and e: Margin of error (generally set at 5%) (Arif, Ayman, Khalid & Humma, 2014). Therefore, 150 samples were projected to be analyzed at MNH. However, only 76 samples met inclusion criteria, including having all the necessary clinical histopathological information, and were analyzed in this study.

3.2.3 Immunohistochemistry (IHC) assay

IHC for Ki67 (Dako Monoclonal Mouse Anti-Human, MIB-1 ready to use), p53 protein (Dako Monoclonal Mouse Anti-Human, DO-7 ready to use), BCL-2 oncoprotein (Dako Monoclonal Mouse Anti-Human, 124), Target Retrieval Solution High pH (50x), Peroxidase-Blocking Reagent (ready-to-use), HRP (ready-to-use), DAB+Chromogen, Substrate Buffer as well as Wash Buffer (20x) were performed on each of 76 tissues section slides manually. Tissue block for staining process were obtained following the protocol below:

(i) **Preanalytic method**: characterized by a fixative sample removed from breast cancer patient in a complex series of chemical solution (10% formal saline for 24 hours) to prevent the basic structure from autolysis and tissue pigmentation in a way that microanatomy and molecular constituents and biomarker could be localized and determined.

   a) **Grossing process**: basically consisted of specimen description (necked eyes), its condition, cut section, selection of proper tissue for microscopic analysis and embedding tissue block in a micro-cassette. Different parameters such as the color specimen, skin and its extend, shape and size of tumor, margin, lymph nodewere assessed for good prognostic under microscope.

   b) **Tissue processing**: was done through dehydration of (70% to 100%) alcohol for water extraction, clearing tissue with xylene then embedding the tissue with molten paraffin wax to increase optical differentiation, hardness of the tissue and help for easy section and IHC staining using Sakura Vacuum Infiltration Tissue Process (VIP6) machine. Thus slide preparation followed through section of paraffin block which was positioned on a microtome for a regular paraffin cut section of 3 μm.

Paraffin slices were left in floatation water bath at 45°C, to eliminate wrinkles and distortions of tissue followed by bonding into a microscopic frosted slides and then under placed in a hot plate at 60°C for 24 hours, for getting rid of the wax before undertaken analytical phase.

(ii) **Analytical method:** Was centered on moisture chamber preparation, application of 10 minutes absolute xylene on the slides for tissue deparaffinization, 10 dips of tissue hydration with 100-95% alcohol and washing slide under water for almost 5 minutes.

IHC staining process was characterized by application of:

a) Diluted Target Retrieval Solution (50x) low pH under pressure cooker for 13 minutes, followed by Wash Buffer's slide (20x) for 5 minutes
b) Peroxidase-Blocking Reagent to the section for 15 minutes, followed by washing slide with Wash Buffer (20x) for 5 minutes
c) Ki-67 (Dako Monoclonal Mouse Anti-Human, MIB-1 ready to use), p53 protein (Dako Monoclonal Mouse Anti-Human, DO-7 ready to use), BCL-2 oncoprotein (Dako Monoclonal Mouse Anti-Human, 124) as primary antibody for 30 minutes. Wash the slide through Wash Buffer (20x) for 5 minutes
d) HRP (Dextran couple with peroxidase molecules and goat secondary antibody) for 30 minutes
e) Washing 3 times the slide with Wash Buffer (20x).
f) Diluted DAB (diaminobenzidine) + Chromogen reagent (1 drop of chromogen reagent to 1 ml of DAB) were applied for 10 minutes on the slides, followed by water wash slide.

Therefore, the slides were counterstained with hematoxylin for 17 dips, followed by washing slides with water for 5 minutes and dehydration in 95% to absolute ethanol, then by cleaned with xylene and mounting through tissue tek-coverslip machine. Moreover, false positive was eluded by a case control test.

(iii) **Post-analytic method:** consisted of slide analysis, control performance evaluation, output description interpretation and outcome reports

3.2.4 Histopathology classification

The histopathological classification of breast cancer was defined in accordance with the worldwide TNM (Tumor, Node, and Metastases) classification of divers' cancer by WHO 2003 (Eble, Tavassoli & Devilee, 2003). However, tumor grade was performed under the

Nottingham grading system. The sub-score of 1 to 3 was consigned on each of the following features: the quantity of tubules formation, nuclear pleomorphism, and mitotic index. Thus, grade III of cancer was given to any patients with a Nottingham score of 8 or 9. Grade II referred to Nottingham scores of 6 and 7, although grade I referred to Nottingham scores of 3, 4, and 5.

3.2.5 Staining assessment

The cutoff point for IHC staining of Ki-67, p53, and BCL-2 varies from literature and studies (Eom, Kim, Lee, Song & Chae, 2016; Inwald *et al.*, 2013; Kim *et al.*, 2015a; Kim *et al.*, 2015b). Thus microscopic evaluation of the slide sections in this study was designed following the common assessment from Eom *et al.* (2016), Inwald *et al.* (2013), Kim *et al.* (2015a) and Kim *et al.* (2015b) through cell counting by recording cells proliferation, as well as the number and intensity of positively stained cells for Ki-67, p53, and BCL-2. Positive slides were considered from 10 to more than 14% of the cells stained, with a score assessment of 2+ (moderate intensity/moderate proliferation) and 3+ (high intensity/high proliferation). In contrast, negative results were observed from the section slides in which less than 10-14% of cells were stained, following a score of 0-1+ (absence of cells staining or low intensity/low proliferation). The IHC evaluation for each studied biomarker was thus conducted as per following Table 1.

Table 1: Microscopic evaluation of IHC staining

Microscopic evaluation and IHC staining assessment	Low biomarkers expression (negative)	High biomarkers expression (positive)
Ki-67	<14% of Ki67 cells stained	>14% of Ki67 cells stained
p53	<10% of p53 cells stained	>10% of p53 cells stained
BCL-2	<10% of BCL2 cells stained	>10% of BCL2 cells stained
IHC assessment	The high intensity of cells staining with low proliferation. Low intensity of cells staining with high proliferation. An absence of cells staining.	High to moderate intensity of cells staining with high to moderate cells proliferation.

3.2.6 Data handling and statistical analysis

Laboratory investigation, histopathological reports, as well as clinical data were entered into structured check-list and Excel spreadsheet software, following a systematic histopathological numbering. Data analysis was performed using SPSS version 23. Likewise, the association of clinic-histopathological factors with prognostic biomarkers and correlation were calculated using correlation test and Multinomial Linear Regression test. The cut-off p-value of 0.05 was used for testing statistical significances.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

Objective 1: To define the clinic-histopathological characteristics and expression levels of Ki-67, p53 and BCL-2 using Immunohistochemistry in patients with breast cancer.

4.1.1 Patient investigation

Retrieval of breast cancer blocks for patients with confirmed breast carcinoma, clinic histopathological information and good morphology were selected for analysis between December, 2016 and December, 2017 at Muhimbili Hospital. Out of 775 cases recorded as admitted or out-patients, 391 (49.4%) Haematoxylin and Eosin (H & E) cases were malignant but only 76 (9.6%) cases (Fig. 4) had good morphology (spatial arrangement of the cells, morphometric characteristics of the nuclei, tubules formation, and number of cancer dividing cells) and complete information met the inclusion criteria. Thus stained for Ki67, p53 and BCL-2 biomarkers using immunohistochemistry (IHC) technique. This sample size of ***n*=76** was therefore used for statistical analysis.

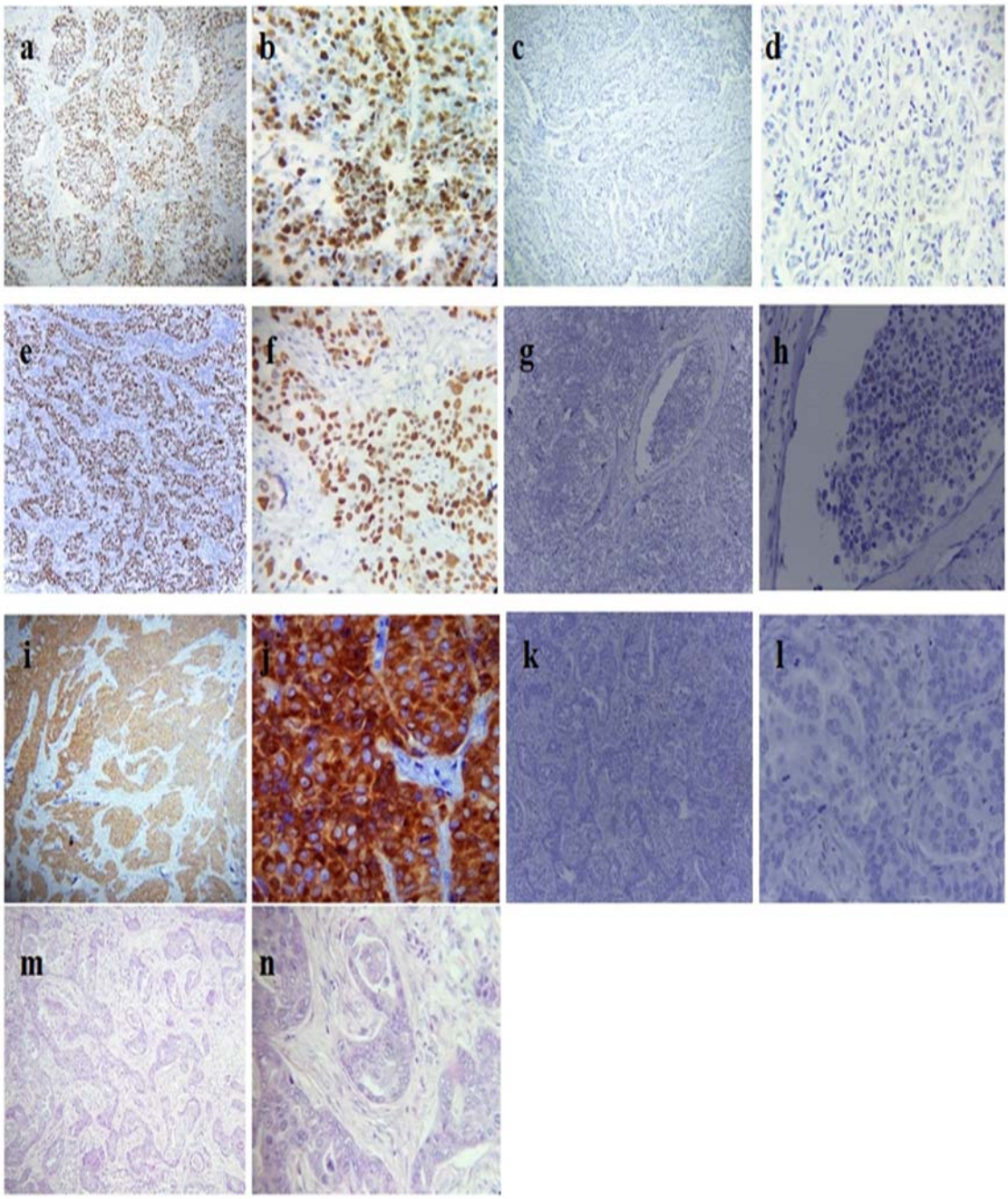

Figure 4: Monograph. a and b: Nuclear positively stained (brown color) for Ki-67 at 10x and 40x hpf respectively; **c and d:** Nuclear negatively stained for Ki-67 at 10x and 40x hpf respectively; **e and f:** Nuclear positively stained (brown color) for p53 at 10x and 40x hpf respectively; **g and h:** Nuclear negatively stained for p53 at 10x and 40x hpf respectively; **i and j:** Nuclear membrane positively stained (brown color) for BCL-2 at 10x and 40x hpf respectively: **k and l:** nuclear membrane negatively stained for BCL-2 at 10x and 40x hpf respectively; **m and n:** H&E staining for infiltrating ductal carcinoma (IDC) at 10x and 40x *hpf* respectively.* **hpf: High-power field.*

4.1.2 Clinic-histopathological and biomarkers outcomes in selected patients

Diagnosis age ranged between 23 to 92 years old with a mean of 51.32 ±14.28 years. Out of 76 patients classified with breast carcinoma, 74/76 samples were pathologically staged, in which 3 (4.1%) and 7 (9.5%) had stage I and II at the initial period of diagnosis in contrast to 36 (48.6%) and 28 (37.8%) presented at late stage in respect to stage III and IV, with a tumor thickness >2cm (T3 and T4) and having more than 3 lymph node (N2 and N3) involved with tumor. However, 36 (49.3%) among them had mostly intermediate grade (grade 2), followed by 25 (34.2%) of high grade (grade 3) breast carcinoma (Table 2).

Furthermore, laboratory investigation revealed that most of the patients with invasive breast carcinoma (IBC) strongly expressed Ki-67 (Ki-67+) and BCL-2 (BCL-2+), with proportions of 65 (85.5%) and 44 (57.9%) respectively. In contrast, high numbers of cases 46 (60.5% of patients) expressed negative response to p53 staining (Table 3). Notably, positive biomarkers expression was mostly expressed by premenopausal patients (<50 years old) (Fig. 5). There was no substantial amount of missing values during the post-analytical phases contrary to the clinic-histopathological side which may potentially lead to misinterpretation in the statistical analysis.

Table 2: Patients and clinic-histopathological characteristics

Characteristic	All(n=76)
Age (years)*	51.32±14.28
T stages	
T1	6 (8.10%)
T2	14 (18.91%)
T3	18 (24.32%)
T4	36 (48.64%)
Nodule status	
N0	13 (17.6%)
N1	10 (13.5%)
N2	30 (40.5%)
N3	15 (20.3%)
Nx	6 (7.4%)
Cancer Grade	
Grade 1	12 (16.4%)
Grade 2	36 (49.3%)
Grade 3	25 (34.2%)
Stage	
Stage I	3 (4.1%)
Stage II	7 (9.5%)
Stage III	36 (48.6%)
Stage IV	28 (37.8%)

Table 3: Distribution of study population with biomarkers

Characteristics	High No.(%)	Low No.(%)	Total of patients No.(%)
Ki-67	65 (85%)	11 (14.5%)	76 (100%)
p53	30 (39.5%)	46 (60.5%)	
BCL-2	44 (57.9%)	32 (42.1%)	

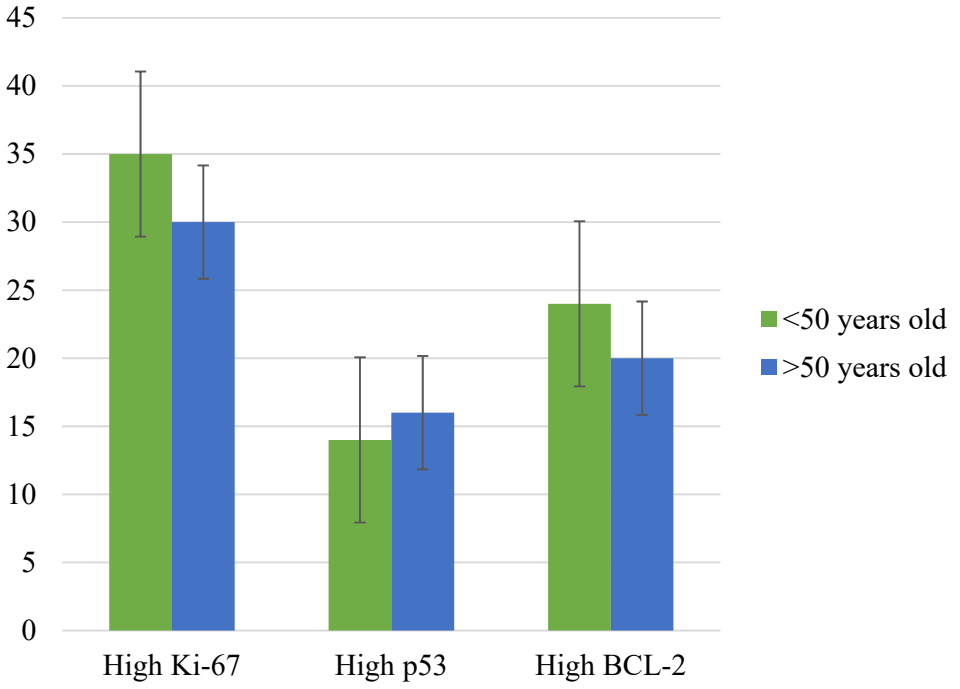

Figure 5: Distribution of Ki-67, p53 and BCL-2 among age-groups

Objective 2: To associate Ki-67, p53 and BCL-2 expression levels with clinical histopathological features.

4.1.3 Relationship between clinic-histopathological characteristics and biomarkers expression

The current findings showed that Ki-67 were highly expressed in stage III and/or IV and grade 2 and/or grade 3 of breast carcinoma (Fig. 6). This is similar with high expression of BCL-2 biomarkers Fig. 7.

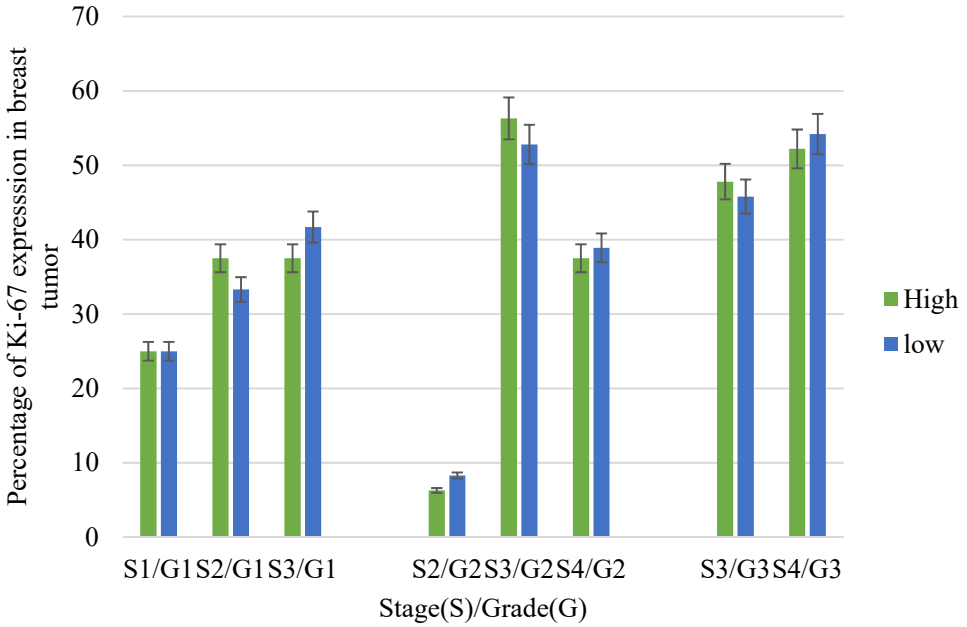

Figure 6: Association between Ki-67 and clinical and histopathological grades

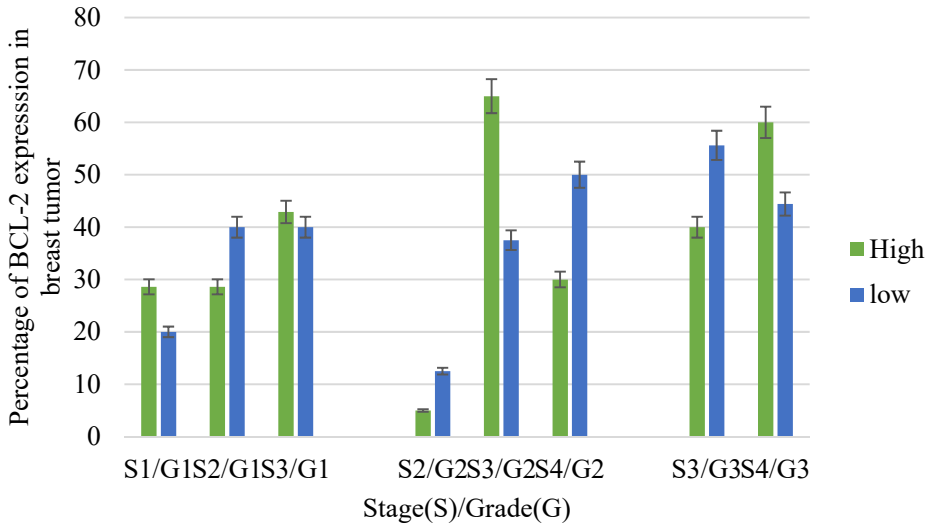

Figure 7: Association between BCL-2 and clinical and histopathological grades

Multinomial logistic regression analysis investigating the relationship between biomarkers and clinico-histopathological characteristics showed that Ki-67, p53 and BCL-2 level ($p>0.05$) were not statistically significantly associated with age, cancer stage, cancer grade, T stage and nodule status. However, these biomarkers could potentially be predictors of poor

outcome (Table 3) when stratified into low and high levels. The odds ratios for associating high Ki-67 expression (as compared to low Ki-67 expression) with cancer grade and tumor stage were; OR 1.254; 95 % CI 0.586–2.683 and OR 1.374; 95 % CI 0.705–2.677 respectively. On the other hand, the odds ratios for associating high BCL-2 exposure (as compared to low BCL-2 expression) with cancer grade and tumor stage were; OR 1.170; 95 % CI 0.512–2.676 and OR 1.533; 95 % CI 0.746–3.149 respectively. Furthermore, with regards to high p53 expression, the odds ratios for associating it with tumor stage were; OR 1.967; 95 % CI 0.834–4.627 while with low p53 expression, the odds ratios for associating it with cancer grade were; OR 1.236; 95 % CI 0.550–2.779. While these odds ratios do contain a null value of 1, which could be because of our smaller sample size (limited by data and inclusion criteria), we still think that these results do indicate a potential promise for these biomarkers to be confirmed in high-powered studies, as tools for evaluating treatment response in individualized therapeutic schemes (Table 4).

Table 4: Association of clinic-histopathological factors with Ki-67, p53 and BCL-2 biomarkers

							95% Confidence Interval for OR	
Low BCL-2[a]		B	Std. Error	Wald	P value.	OR (Odd Ratio)	Lower Bound	Upper Bound
High Ki-67	Age	-0.008	0.017	0.2356	0.627	**0.991***	0.958	1.025
	Cancer stage	-0.344	0.527	0.4279	0.512	0.708	0.252	1.990
	Cancer grade	0.226	0.388	0.3406	0.559	**1.254***	0.586	2.683
	T stage	0.318	0.340	0.8763	0.349	**1.374***	0.705	2.677
	Nodule status	-0.089	0.233	0.1463	0.702	0.914	0.579	1.444
	Histologic subtype	-0.224	0.241	0.8666	0.351	0.798	0.497	1.282
Low Ki-67	Age	0.048	0.030	2.5518	0.110	1.049	0.989	1.114
	Cancer stage	-0.275	0.853	0.1042	0.7467	0.759	0.142	4.041
	Cancer grade	-0.854	0.740	1.3330	0.248	0.425	0.099	1.815
	T stage	-0.113	0.536	0.0444	0.833	0.893	0.312	2.554
	Nodule status	0.167	0.404	0.1715	0.678	**1.182***	0.535	2.609
	Histologic subtype	-0.025	0.371	0.0048	0.944	0.974	0.470	2.020
High p53	Age	0.009	0.019	0.2358	0.627	1.009	0.970	1.050
	Cancer stage	-0.079	0.626	0.0161	0.898	0.923	0.270	3.154
	Cancer grade	0.002	0.484	1.8117E-05	0.996	1.002	0.387	2.588
	T stage	0.675	0.436	2.3904	0.122	**1.965***	0.834	4.627
	Nodule status	0.011	0.271	0.0018	0.965	1.011	0.594	1.720
	Histologic subtype	-0.730	0.694	1.1066	0.292	0.481	0.123	1.878
Low p53	Age	-0.006	0.018	0.1446	0.703	0.993	0.958	1.029
	Cancer stage	-0.495	0.561	0.7800	0.377	0.609	0.202	1.829
	Cancer grade	0.2121	0.413	0.2651	0.606	**1.236***	0.550	2.779
	T stage	0.065	0.356	0.03384	0.853	1.067	0.531	2.146
	Nodule status	-0.063	0.252	0.0627	0.802	0.938	0.572	1.540
	Histologic subtype	-0.145	0.230	0.3980	0.528	0.864	0.550	1.358
High BCL-2	Age	-0.001	0.018	0.0054	0.941	0.998	0.963	1.035
	Cancer stage	-0.542	0.559	0.9396	0.332	0.581	0.194	1.740
	Cancer grade	0.157	0.421	0.1394	0.708	**1.170***	0.512	2.676
	T stage	0.427	0.367	1.3573	0.243	**1.533***	0.746	3.149
	Nodule status	-0.071	0.249	0.0821	0.774	0.930	0.570	1.518
	Histologic subtype	-0.456	0.326	1.9561	0.161	0.633	0.334	1.200

[a]The reference category

*OR>1 has causative effect on the cancer patients

4.1.4 Antagonistic expression among Ki-67, p53 and BCL-2

Throughout the post-analytical microscopic phase, it was interesting to find that Ki-67, BCL-2 and p53 were antagonistically expressed for any combination of two of them. This raised the question whether the high levels expression of p53 influenced the level expression of BCL-2 and Ki-67. Statistically tested, p53 showed significant correlation with BCL-2 (Pearson's correlation=-0.238, *p*-value=0.038) with a Covariance = -0.058. In the same way BCL-2 was significantly correlated with Ki-67 expression (Pearson's Correlation=0.331, *p*-value=0.004) with a Covariance=0.058. However, no significant correlation has been observed between Ki-67 and p53 (Table 5).

Table 5: Antagonistic biomarkers expression

		Ki-67 biomarker	**p53 biomarker**	**BCL-2 biomarker**
Ki-67 biomarker	Pearson Correlation	1	.103	.331**
	Sig. (2-tailed)		.377	**.004**
	Sum of Squares and Cross-products	9.408	1.342	4.368
	Covariance	.125	.018	**.058**
	N	76	76	76
p53 biomarker	Pearson Correlation	.103	1	**-.238***
	Sig. (2-tailed)	.377		**.038**
	Sum of Squares and Cross-products	1.342	18.158	-4.368
	Covariance	.018	.242	**-.058**
	N	76	76	76
BCL-2 biomarker	Pearson Correlation	**.331****	-.238*	1
	Sig. (2-tailed)	**.004**	.038	
	Sum of Squares and Cross-products	4.368	-4.368	18.526
	Covariance	**.058**	-.058	.247
	N	76	76	76

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

4.2 Discussion

Limited molecular bio-markers are currently in clinical use for BC management in Tanzania as only ER, PR, and HER-2 are used as HR for guiding hormonal treatment of BC., However, Ki67, p53 and BCL2 are not used for BC classification or as prognostic bio-markers for breast cancer management at Muhimbili National Hospital or OCRI. Previous studies have demonstrated an exponential increase in numbers of research on bio-markers, and regarding new technique of early diagnostic and predictive factors for breast cancer that could enhance accuracy treatment.

As heterogeneous disease with various biomarkers expression, BC was classified in this study as luminal A (ER+, PR+ HER2- and low level of Ki-67), luminal B (ER+, PR+ HER2+/- and high level of Ki-67), triple negative and enriched HER2 (Liu *et al.*, 2017). Similarly, Dumay *et al.* (2013) suggested that p53 could be more classified in luminal B rather than luminal A (Dumay *et al.*, 2013). Besides this classification, most of the patients attending MNH with BC were obviously tested for hormonal receptors, as well as HER-2, and they were followed by a general application of endocrine therapy with Tamoxifen at Ocean Road Cancer Institute (Mabula *et al.*, 2012; Mwakigonja, Lushina & Mwanga, 2017) without any complementary biomarkers investigation, which may lead to drug resistance or non-responding treatment.

The current research was conducted on 76 cases retrieved from December 2016 and December, to determine the correlation between clinical and histopathological prognostic factors with Ki-67, p53 and BCL-2 bio-markers expression in selected BC's patients at MNH, Tanzania.

However, current findings showed that 86.8% of patients presented with infiltrating ductal carcinoma (IDC), followed by 6.6% of lobular carcinoma (ILB) and others. Nonetheless, positive biomarkers expression was mostly expressed by premenopausal patients (<50 years old) probably due to a mechanism of upregulation regarding to hormonal receptors expression. The mean age of 51.32 and diagnosis age ranged from 23 to 92 years old. This representative age-group is generally in unison with related studies (Hwang *et al.*, 2018; Inwald *et al.*, 2013; Mbonde *et al.*, 2001).

Numerous studies have described Ki-67 as key biomarker more significantly associated with malignant tissue rather than normal tissue. However, in the recent study, the largest number of patients had tumors which were Ki-67+, which corresponded mostly to luminal B

subtypes. These tumors usually had an intermediate grade; negative p53 (p53-) status, and high levels of BCL-2(BCL-2+) expression. Thus many studies claim Ki-67 as a predictor biomarkers for neo-adjuvant response (endocrine therapy) (Dai, Xiang, Li & Bai, 2016).

Based on the relationship between biomarkers expression and clinic-histopathological features, results demonstrated that some findings were not obvious in our study, because of non-significant statistical results that are most likely due to the low power of our study. In this study, bivariate analyses revealed only marginal statistical significance but multinomial analysis did not reveal any significance. As both Ki-67/BCL2 and p53 are implicated in cell proliferation and apoptosis, they play an essential role in defining tumor growth and may more accurately help to define high-risk patients. Hence, patients with high Ki-67, low p53 and high BCL-2 are more likely to be at high risk of having poor prognosis compared to patients with low BCL2 in this study, which needs to be confirmed in bigger studies as suggested in this study.

Comparing this study with Awadelkarim *et al*. (2012), Shapochka *et al*. (2013) and Angel *et al*. (2014), our findings herein swerve from these authors. While they found a statistically significant relationship between biomarker expression and clinical histopathological features, our study found no such statistically significant association, most likely due to limited sample size with low power. Following a research study by Angel *et al.* (2014), 251 cases were investigated to establish the affiliation amongst tumor size, lymph node status and immunohistochemical expression of Ki-67, p53, and BCL-2 in patients with BC. Their research demonstrated a significant association between tumor size with advanced histological grade, high cell proliferation (Ki-67 expression) and p53. However, with regards to age, their study showed significant association of tumor size among women over 70 years old, which is contrary to our findings, where no statistically significant association was observed across the age groups with biomarkers. Shapochka *et al.* (2013) as well reported the association between Ki-67 with tumor grade but their conclusion regarding the link between p53 and tumor grade was not observed in this current study. Further, Filip *et al.* (2008) revealed no significant relationship among BCL-2 positivity with tumor grade and primary tumors, which concurs with our findings herein (Filip *et al.*, 2008).

During the post-analytical microscopic phase, it was interested in this current study to find that Ki-67, BCL-2, and p53 were antagonistically expressed for any combination of two of them. This begged the question whether the high expression levels of p53 influenced the

expression levels of BCL-2 and Ki-67, and vice versa. When statistically tested, p53 showed significant negative correlation with BCL-2. In the same way, BCL-2 significantly correlated with Ki-67 expression. However, no significant correlation was observed between Ki-67 and p53. p53 is a tumor suppressor protein that is encoded by TP53 tumor suppressor gene, which is a transcription factor that regulates genes involved in cellular processes when activated by cellular stress responses, which include cell cycle and apoptosis. So p53 is pro apoptotic during cellular stress. Mutated p53 is overexpressed in several tumors, including breast cancer, where it is associated with higher tumor differentiation, adverse estrogen and progesterone receptors, or HER2 status. BCL-2 protein, on the other hand, is encoded by the BCL-2 gene and plays an anti-apoptotic role by inhibiting cell death, which results in prolonged cell survival. BCL-2 is overexpressed in many cancers and contributes to tumor initiation, progression, and resistance to therapy. In many cancers, BCL-2 overexpression contribute in the initiation, development, and treatment resistance of tumors. The two antagonizing roles of p53 and BCL-2 proteins (Nalwoga, 2010) may therefore explain the negative correlation between the two as observed in this current study.

Antagonistic effect among p53 and BCL-2 expression was reported by Ruth, Ella, Douglas and Donald (1997) as well as Subrata, Massimo, Maria, Silvia and Carlo (1994) confirming the significant correlation between BCL-2 and p53. They stipulated that the level of BCL-2 in cells is regulated by the p53 protein on the principle of feedback. Further mechanistic studies on the roles of p53 and BCL-2 signaling pathways in BC are thus warranted (Ruth *et al.*, 1997; Subrata *et al.*, 1994). With reference to the positive correlation between BCL-2 and Ki-67, most of the studies have shown the correlation between BCL-2 and hormonal receptors expression in contrast to Ki-67. Shapochka *et al*. (2013) affirmed in their investigation that the level of tumor proliferation was inversely correlated with expression of hormonal receptors and BCL-2. Nevertheless, this theory needs further research to support it. Besides, this study did not cover the assessment of hormonal receptors (ER and PR) and HER2 in association with our new predictive biomarkers, which could give us more information on patient survival.

4.3 Study limitations

This study did not cover a large number of samples size due to logistic constraints. Numerous files were figured neither as archives nor in the system, meaning the system of cancer registry and availability of database for cancer patients at the MNH and Tanzania are still very weak. This all contributed to our limited sample size. Yet, we still believe that the

current results are essential for further research work in understanding the possible diagnostic and prognostic functions of the studied biomarkers, and their respective mechanisms.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Breast cancer is a heterogeneous disorder owing to molecular and histologic modifications with regard to tumor biology, clinical behavior, prognosis, and reactions to therapy. In this line, currents study aimed to explore novel biomarkers that could be interesting in understanding and management of the disease in Tanzanian women. Uppermost levels of Ki-67 and BCL-2 were expressed by patients under 50 years' old which is the most productive period of women, contrary to the last research finding conducted locally. The studied biomarkers herein found that Ki-67, p53 and BCL-2 were highly associated with some reported clinical-histopathological features and poor outcomes. As these biomarkers' expression was linked to diverse subtype, including luminal A and Luminal B, this suggested that the patients expressing those biomarkers in this study could be sensitive to an endocrine therapy. Conclusively, there is evidence of correlation between the studied markers with CH features making these markers potential tools for evaluating treatment response in individualized therapeutic schemes.

5.2 Recommendations

From the conclusion, the following recommendations were made:

(i) Based on the hormonal receptors targeting BC treatment in Tanzania, it is suitable to increase the variety of research on other markers to individualize Tanzania therapy systems promptly

(ii) In order to positively affect the lives of individual breast cancer patients through personalized therapy systems, breast-cancer management in Tanzania should discover a manner to improve the pathological facilities, including immunohistochemistry for screening biomarkers in a regular cancer management process.

(iii) A study with bigger sample size is thus recommended to validate the findings from this study, including studying the mechanisms involved in the observed differences among association of markers' expressions and respectively studied clinic pathological characteristics.

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APPENDICES

Appendix 1: Check list

Study area: Muhimbili National Hospital- Tanzania

File Number: **Patient's name:** **Age:**

Clinico pathological parameters	Sub Characteristics	Ki-67 expression		p 53 Expression		BCL2 Expression		Supplementary information
Tumor site	Lobule ☐ Duct ☐	**low**	**High**	**Low**	**High**	**Low**	**High**	
Tumor size	T1 T2 T3 T4							
Clinical Tumor Stages	I II III IV							
Nodal Status	N0 N1 N2 N3							
Metastasis	M0 M1 Mx							
Histological grade	G1 G2 G3							

Appendix 2: Ethical clearance

THE UNITED REPUBLIC OF TANZANIA

National Institute for Medical Research
3 Barack Obama Drive
P.O. Box 9653
11101 Dar es Salaam
Tel: 255 22 2121400
Fax: 255 22 2121360
E-mail: ethics@nimr.or.tz

Ministry of Health, Community Development, Gender, Elderly & Children
University of Dodoma, Faculty of Arts and Social Sciences
Building No. 11
P.O. Box 743
40478 Dodoma

NIMR/HQ/R.8a/Vol. IX/2707

01st March 2018

Hidaya Mansour
C/o Dr. Elingarami S. Nkya
Nelson Mandela African Institute of Science and Technology
P. O. Box 447
Arusha

RE: ETHICAL CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled: Molecular classification of breast cancer via immunohistochemistry at Muhimbili Referral Hospital - Tanzania (Mansour H. *et al*) whose local investigator is Dr. Elingarami S. Nkya of Nelson Mandela African Institute of Science and Technology has been granted ethical clearance to be conducted in Tanzania.

The Principal Investigator of the study must ensure that the following conditions are fulfilled:

1. Progress report is submitted to the Ministry of Health, Community Development, Gender, Elderly & Children and the National Institute for Medical Research, Regional and District Medical Officers after every six months.
2. Permission to publish the results is obtained from National Institute for Medical Research.
3. Copies of final publications are made available to the Ministry of Health, Community Development, Gender, Elderly & Children and the National Institute for Medical Research.
4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine as per NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Site: Dar es Salaam.

Approval is valid for one year: 01st March 2018 to 28th February 2019.

Name: Prof. Yunus Daud Mgaya

Signature
CHAIRPERSON
MEDICAL RESEARCH
COORDINATING COMMITTEE

Name: Prof. Muhammad Bakari Kambi

Signature
CHIEF MEDICAL OFFICER
MINISTRY OF HEALTH, COMMUNITY DEVELOPMENT, GENDER, ELDERLY & CHILDREN

CC: RMO of Dar es Salaam
DMOs/DEDs of selected districts

Appendix 3: Research permission from Muhimbili National Hospital

MUHIMBILI NATIONAL HOSPITAL

Cables: "MUHIMBILI"
Telephones: +255-22-2151367-9
FAX: +255-22-2150534
Web: www.mnh.or.tz

Postal Address:
P.O. Box 65000
DAR ES SALAAM
Tanzania

In reply please quote: MNH/TRC/Permission /2018/302 22th March, 2018

Head,
Central Pathology Laboratory,
Muhimbili National Hospital

RE: PERMISSION TO COLLECT DATA AT MNH

Name of Student	Ms. Hidaya Mansouri
Title	"MOLECULAR CLASSIFICATION OF BREAST CANCER VIA IMMUNOHISTOCHEMISTRY AT MUHIMBILI NATIONAL HOSPITAL REFERRAL HOSPITAL-TANZANIA".
Institution	Nelson Mandela African Institution of Science and Technology
Co- Supervisor	Dr. Elingaami S. Nkya Dr. Emmanuel Mpolya
Period	22/03/2018 to 30/09/2018

The above named student has been permitted to collect data for the above study.
Please ensure that the researcher abide to the ethical principle and other conditions.

Sincerely,

HEAD, TEACHING RESEARCH & CONSULTANCY UNIT
MUHIMBILI NATIONAL HOSPITAL
P. O. BOX 65000
DAR ES SALAAM

Dr. Faraja Chiwanga
Head of Teaching, Research and Consultancy Coordination Unit

c.c. DCSS
c.c. Ms. Hidaya Mansouri

All Correspondences to be addressed to the Executive Director